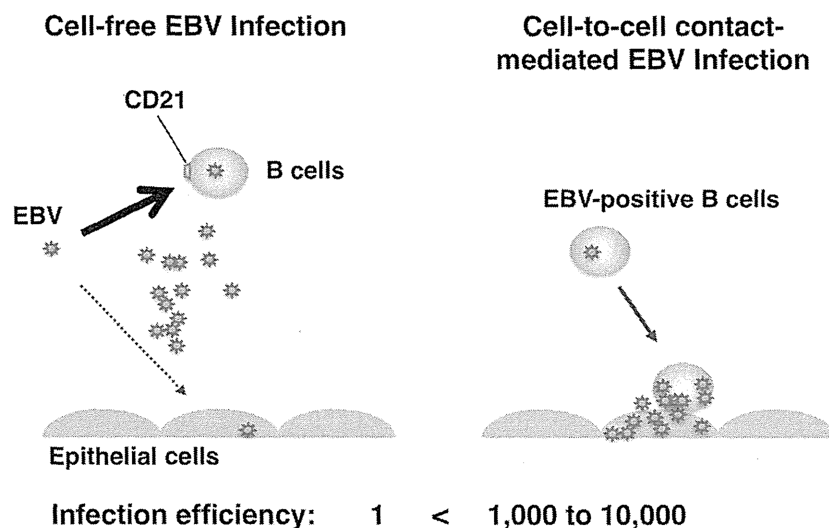
Figure 3. Cell-to-cell contact-mediated EBV transmission to epithelial cells. In cell-free infection, EBV preferentially infects B cells using CD21 receptor. EBV also infects CD21-negative epithelial cells as part of its normal life cycle, however much less efficiently. EBV transfer mediated by cell-to-cell contact with B cells increases the infection efficiency 1,000 to 10,000-fold compared with cell-free virus infection.



Several lines of evidence indicate that EBV infection into epithelial cells is mainly mediated by cell-to-cell contact [34,51-55]. The rate of EBV infection in epithelial cells is 10^3-fold higher by co-culturing with EBV-positive B cells than by cell-free infection [34,51,55]. Moreover, most EBV virions are retained on cell surfaces after binding to primary B cells and transferred to epithelial cells, resulting in the 10^3 to 10^4-fold increase of infection compared with cell-free virus infection [53,54].All these studies support a model that EBV-infected B cells migrating into the epithelial stroma or intraepithelial space contribute to the efficient EBV transmission into epithelium via cell-to-cell contact (Figure 3). The detailed molecular mechanisms of cell-to-cell EBV transmission remain unclear. Shannon-Lowe *et al.* showed that EBV virions loaded on the surface of primary B cells facilitate the formation of a virological synapse (VS)-like intercellular conjugation between B cells and co-cultured epithelial cells [53,54]. The VS is a tight adhesive junction across which virus can be efficiently transferred from virus-infected cells to non-infected target cells without cell-cell fusion [56].An important role of an EBV glycoprotein, BMRF2 in the cell-to-cell spread of EBV in polarized oral epithelial cells has been proposed [39,40]. The mucosa of oropharyngeal and nasopharyngeal regions are known to be heavily filtrated by lymphocytes, suggesting that cell-free virus in the saliva could first bind to the surface of B cells and then efficiently transfer to pharyngeal epithelial cells through a cellular conjugate between B cells and epithelial cells.

The detailed molecular mechanism by which EBV infects epithelial cells still remains unclear. Some key factors involved in viral attachment, membrane fusion, and cell-to-cell contact-mediated viral transmission, have been identified [48,49]. However, no cellular receptors for EBV infection have yet been identified on epithelial cells.Also very little insights have been provided for a conceptual understanding of viral entry mechanism into epithelial cells. Further investigations are still required.

4. Viral Genes and Carcinogenesis

4.1. Models of EBV infection of gastric epithelial cells

EBV infects both B lymphocytes and epithelial cells, since the virus is discovered in BL cells, HL cells, NPC cells, and EBVaGC cells. Experimental EBV infection to B cells is very efficient, since EBV uses high affinity receptor, CD21 for its entry [16]. However, epithelial cells are CD21-negative and infection of epithelial cells could not be achieved for a long time, exceptionally when CD21 expression was overcome by gene transfer [33,57].

We have clearly proved direct infection of human gastric epithelial cells by EBV [50]. The infection was achieved by using a recombinant EBV with a selectable marker gene [58,59], but without any operations, such as introduction of the CD21 gene. In our study, epithelial cells were negative for CD21 and the infection was not blocked by anti-CD21 monoclonal antibody [50]. We have next showed that efficient transfer of EBV to epithelial cells by mixed culture with recombinant EBV producing B cells [34]. Other than experimentally EBV-infected cells, SNU-719 cell [60] and KT cell [61] are few cells retaining the same clonal EBV genome and EBV gene expression pattern of latency I as the original tumor biopsy. EBV-harboring epithelial cells are difficult to propagate *in vitro* and in animal models.

4.2. Growth promoting effects of EBV

The KT cell is a good *in vivo* model of EBVaGC and expresses high IL-1ß compared with EBV-negative gastric tumor cells [62]. Primary cell cultures from healthy gastric mucosal biopsies were infected with recombinant EBV [63]. The established cells expressed Qp-driven EBNA 1, EBER, BART, and LMP2A, similar to EBVaGC. The EBV-positive clones showed rapid proliferation and p53 overexpression, and exhibited anchorage independence in colony formation assay.

Growth promotion by EBV infection was also observed in EBV-infected NU-GC-3 cells through secretion of insulin-like growth factor (IGF)-1 as an autocrine growth factor [64]. It has been shown that EBERs play an oncogenic role by inhibition of apoptosis [65,66] and IGF-1 induction [67]. The oncogenic role of other genes, such as BARF1 [68] and LMP2A [69] has been reported.A recent report showed that EBV infection affected miRNA expression [70]. While no consensus exits as to the exact mechanism by which EBV promotes EBVaGC, the establishment of EBVaGC cell lines and the development of an *in vitro* model represent significant progress towards this goal.

5. Virus and Host Interactions at Molecular Level

5.1. DNA Hypermethylation in EBV and Host Genomes

A number of CpG islands in the promoter region of an tumor suppressor gene have been methylated in cancer cells than in normal cells [71]. Expression of many genes, such as *p16* and *RUNX3*, is suppressed in stomach cancer owing to promoter methylation [72,73]. Promoter hypermethylation is especially frequent in EBVaGC [74-76]. Methylation of promoter region in *APC*, *p16*, *MINT1*, *MLH1*,

TP73, and *HOXA10* [14,77,78] has been specifically observed in EBVaGC (Table 1).A large-scale analysis revealed that *CXXC4*, *TIMP2*, and *PLXND1* are specifically methylated in EBVaGC [79]. Down regulation of CXXC4, a suppressor of the Wnt pathway, promotes tumor cell proliferation and invasiveness [80]. Down regulation of TIMP2, a suppressor of metalloproteinase, inhibits tumor cell metastasis [81]. Methylation of similar genes has been reported in cancers associated with hepatitis B or C infection [82,83], suggesting that a common mechanism may underlie the formation of infection-associated cancers.

Table 1. DNA hypermethylation in EBVaGC.

Name	Function	Reference
APC	Tumor suppressor, regulate ß-catenin signal	14
p16, p14	Tumor suppressor, regulate cell cycle	14, 77
TP73	Tumor suppressor, regulate apoptosis	77, 78
CXXC4	Suppressor of Wnt signal	79
TIMP2	Inhibitor of metalloproteinase	79

However, the precise molecular mechanism of host DNA methylation during the early stage of EBV infection of the gastric epithelium is not fully understood. It is reported that LMP2A induces the phosphorylation of STAT3, which activates DNA methyltransferase 1 (DNMT1) transcription and causes PTEN expression loss through CpG island methylation of the *PTEN* promoter [69].Although LMP2A is expressed in substantial cases of EBVaGC [84], EBVaGC patients are usually negative for LMP2A antibody [85]. Constitutive overexpression of DNMT1 has been observed in EBV-infected gastric epithelial cells that do not express LMP2A significantly [69]. Further investigations using EBV mutants of LMP2A [86] may uncover its precise molecular mechanism.

5.2. miRNA and Carcinogenesis

miRNAs are endogenous 18 - 25 nt RNAs and play important gene-regulatory roles in eukaryotic cells via posttranscriptional repression of gene expression. miRNA targets 3′-untranslated region (UTR) elements of mRNA and mediates mRNA decay through degradation of polyA (RNA silencing: RNAi) [87]. Different expression patterns of miRNA from normal tissues were expected in cancer. Several miRNAs and non-coding RNAs have been found to have links with some types of cancer and are referred as "oncomirs". This is because miRNAs have a role as oncogenes when they target tumor suppressor genes. On the other hand, miRNAs are tumor suppressors when they target oncogenes [88].

A clonal EBV infection has been found in EBV-associated epithelial tumors, such as NPC and EBVaGC [9,89]. EBV encodes a large number of miRNAs [90]. Up to 25 pre-miRNAs are encoded in the BHRF1 and BART regions of the genome, which result in four mature BHRF1 miRNAs and 40 BART miRNAs [91].A prototypic EBV strain B95-8 is known to have 11 kbp deletion in BART region. However, B95-8 virus can be produced in large amount and possesses prominent B cell transformation ability. Moreover, a recombinant virus lacking entire region of BART region was still

able to infect and transform B cells [92]. These findings indicate that BART transcripts are not required for B cell transformation and neglected the importance of BART for a long period. Cai *et al.* identified 13 *BART* miRNAs in the region of B95-8 deletion [91].

Table 2. EBV-derived miRNAs and their target genes.

Name	Target	Reference
BHRF1-1	GUF1, SCRN1	101
miR-BART1	LMP1 (EBV), CLEC2D, LY75, SP100, DICER1, MICB	93, 101
miR-BART2	BALF5 (EBV)	94
miR-BART3	DICER1, MICB	101
miR-BART5	PUMA, LMP1 (EBV)	96, 100
miR-BART6	DICER1	97
miR-BART10	BHRF1 (EBV)	100
miR-BART13	CAPRIN2	100
miR-BART17	LMP1 (EBV)	93
miR-BART19	LMP1 (EBV)	100
miR-BART22	LMP2A (EBV)	95
BARTs	BIM	98

EBV-encoded BART miRNAs target the 3'-UTRs of viral genes, such as LMP1, BALF5, and LMP2A genes, and negatively regulate expression of these viral genes [93-95]. On the other hand, EBV miRNAs repress cellular proteins, which include p53 up-regulated modulator of apoptosis (PUMA), DICER1, and BIM [96-98] (Table 2). Preservability of the 3'-UTR domain of the target gene between species is a common feature of mammalian miRNA. However, since EBV preferentially infects humans, some EBV miRNAs characteristically target only human gene and not genes for other mammals. EBV miRNAs have another characteristic feature, some of which binding motifs are found only on EBV genome and not preserved on other viral genomes [97,99]. In order to identify EBV miRNA targets, a transcriptome-wide identification of miRNA binding sites has been performed between EBV-negative and EBV-positive cell lines [99-101].

Biological significance of viral miRNA in EBV-infected cells was searched using EBV recombinants. In these recombinants, mutations were systematically introduced in EBV's precursor miRNA transcripts to prevent their subsequent processing into mature viral miRNAs. Phenotypic analyses of miRNA mutants revealed that the viral miRNAs contribute to EBV-associated cellular transformation rather than regulation of viral lytic replication [102].

There are two complicated stories in EBV-infected cells. One is that expression of viral latent genes induces cellular miRNAs. Viral LMP1 induces human miR-146a and miR-155 expression [103-105].And excessive miR-155 expression is known to form B lymphoma [106]. Two, infection of EBV conversely suppresses entire host cell miRNA expression [70,107]. Since down-regulation of the cellular microRNA family miR-200 causes epithelial-mesenchymal transition (EMT), this phenomenon must be an important step in the process of malignant transformation of both EBVaGC

and nasopharyngeal carcinoma cells. The precise mechanism for dysregulation of host miRNA by EBV infection needs to be clarified. It is known that low expression level of miRNA processing enzymes, DICER1 and DROSHA, is well correlated with tumor progression in many cancers [108]. We have shown that the expression level of human DICER1 is also lower in EBV-infected cells than in non-infected cells [97,101]. These findings suggest that progression of EBV-associated tumor is possibly regulated by EBV through regulation of viral and host miRNA expression. However, most of the studies presented in this section are performed using BL cells. Further study using EBV positive gastric epithelial cells is required.

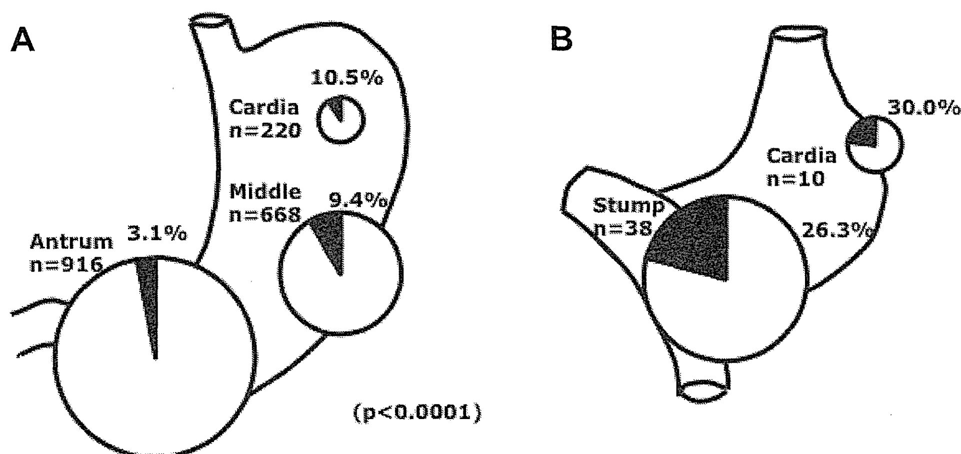
6. Diagnosis and Treatment of EBV-associated GC

6.1. Procedure

EBER1-ISH is a most sensitive method to identify EBV infection.Application of EBER1-ISH to gastric mucosal biopsy samples from patients who have undergone upper gastrointestinal endoscopy is very useful to make diagnosis of EBVaGC before treatment. Patients with EBVaGC had elevated serum antibodies against EBV early antigen and EBV capsid antigen. However, EBNA1 antibody titers did not show significant difference between patients and healthy counterparts [9].

Yanai *et al.* examined 124 gastric carcinomas from 117 patients using EBER1-ISH.Among them, twelve tumors (9.7%) were identified as EBV associated [109].An interesting feature of EBVaGC is its predominance in the non-antrum part of the stomach (Figure 4A). EBVaGC appears as a superficial depressed- or ulcerated-lesion in the upper part of the stomach. Histology of EBVaGC is mainly diffuse-type carcinoma rich in lymphocyte infiltration (gastric carcinoma with lymphoid stroma). Endoscopic ultrasonography revealed a hypoechoic mass in the third layer, reflecting submucosal nodules [110].

Figure 4. EBVaGC by site in the stomach.A**.** Distribution of EBVaGC. EBV prevalence was more frequent in the cardia and middle stomach than in the antrum, where over half of EBV-negative gastric cancers were located. **B.** EBV involvement in remnant cancer by site. Note that frequency of EBV infection in the non remnant cardiac cancer was 10.5% [22].



Gastric remnant cancer (gastric stump cancer) after distal gastric resection for benign disease, such as refractory gastric or duodenal ulcer disease or recurrent ulcer with gastric outlet obstruction, remains a substantial clinical concern, because the incidence of remnant cancer is still increasing (Figure 4B) [111].A high prevalence of EBV involvement (25 to 41.2%) in gastric remnant carcinoma has been reported. High cell proliferation activity in the epithelium has been reported. The reflux of bile and pancreatic juice is considered to cause regenerative atypia and cell proliferation in epithelial cells [112].Atrophic change of remnant gastritis in Billroth-II anastomoses was associated with EBV-positive gastric remnant carcinoma with high incidence [113].

6.2. Prognosis

To date there is no specific therapeutic method for EBVaGC. Since the frequency of undifferentiated type of cancer is high in EBVaGC, most of the tumor is surgically resected. CpG island methylation of the promoters of various tumor-related genes plays important roles in the development and progression of gastric cancer [16]. Statistical analysis showed that promoter hypermethylation is more frequently observed in EBVaGC than in EBV-uninfected gastric carcinoma [69,74-76]. Medical treatment with a demethylation agent, which induces lytic EBV infection in latently EBV infected cells, may lead to a lysis of cancer cells. This approach could be applied to the medical treatment of EBVaGC, since methylation of the tumor suppressor gene is also a key abnormality in EBVaGC [12,114,115].

Most of the previous reports did not observe any prognostic difference between EBV-positive and -negative gastric cancer [21]. However, early EBVaGC has the low frequency of lymph node metastasis even in submucosal type. Partial medical treatment, such as endoscopic treatment, can be adapted to such a case. The authors have experienced a case of early EBVaGC with submucosal invasion, in which palliative endoscopic treatment was performed. Recurrence was not observed in this case for more than four years [116].A clinicopathological study in the Netherlands mentioned that EBVaGC accompanied lymph node metastasis in significantly lower frequency than EBV-negative stomach cancer. In the study, EBVaGC cases showed a better prognosis than negative cases [117].

7. Conclusion

Considerable studies suggest that EBV contributes to cell proliferation and survival, and may directly contribute to the development of EBVaGC through these effects. EBV affects multiple host proteins and pathways that normally promote apoptosis and regulate cell proliferation. However, the underlining molecular mechanisms of these effects are complex and certainly affecting each other.

In another aspect, inflammation of the stomach will recruit EBV-infected B-lymphocytes in the vicinity of gastric epithelia and may increase the frequency of EBV infection of epithelia. Differences in individual inflammatory response by either genetic and/or environmental effect, such as a predisposing loss of ARID1A in epithelial cells before EBV infection [118] or single nucleotide polymorphisms of promoter region of interleukin-10 and/or tumor necrosis factor-α [119], possibly affect the oncogenic pathway to EBVaGC.

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Conflict of Interests

The authors declare no financial or commercial conflict of interest.

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Regulatory mechanisms of nucleic acid-mediated innate immune responses in the tumor microenvironment

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Keywords: dendritic cells, HMGB1, innate immunity, nucleic acids, TIM-3

Abbreviations: DAMPs, danger-associated molecular patterns; DC, dendritic cells; HMGB1, high mobility group box 1; TIM-3, T cell-immunoglobulin mucin protein 3; TLRs, Toll-like receptors

We identified novel mechanisms whereby TIM-3 suppresses innate immunity as induced by nucleic acids. Interaction of TIM-3 with HMGB1 inhibits the recruitment of nucleic acids to the endosomal compartment of dendritic cells, impairing the transduction of innate immune signals. Thus, TIM-3 is an effective target for enhancing the immunogenicity of nucleic acids in the context of cancer therapy.

Innate immunity serves as a well-organized pattern recognition system for infectious and stress-induced components within inflammatory microenvironments. In particular, nucleic acids derived from infectious agents or dying host cells are detected by pattern-recognition receptors such as Toll-like receptors (TLRs) and cytosolic sensors for DNA and RNA, leading to the induction of cytokines and other proinflammatory mediators that are essential for innate immune responses.[1]

Discrimination between foreign and self nucleic acids serves as a key mechanism protecting the host from pathogens while maintaining tissue homeostasis, as exposure of self-nucleic acids to the innate immune system frequently results in severe inflammation and autoimmune reactions.[2] However, several studies have unveiled mechanisms whereby self nucleic acids gain access to components of the innate immune system. Endogenous proteins released from inflammatory environments, such as danger-associated molecular patterns (DAMPs), preferentially interact with self nucleic acids. Thus, the formation of complexes with DAMPs enables nucleic acids to gain access to the endosomal compartment, in which receptors from the innate immune system recognize nucleic acids and orchestrate proinflammatory responses.[3] In addition, recent studies reveal that exogenous nucleic acids mediate immunogenic activities by interacting with pattern recognition systems. For example, DNA released from dying host cells has a key role in triggering the adjuvant effects of aluminum, thus facilitating dendritic cell (DC) migration and antigen-specific T-cell responses.[4] Moreover, cellular damage as induced by UV irradiation results in the structural modification of self RNAs and hence in the delivery of innate immune signals via TLR3.[5] Thus, nucleic acids generated from host cells have the potential to activate innate immunity under various pathological situations.

Transformed cells are assumed to be a mixture of self and "non-self" nucleic acids, the latter being a result of tumor-associated mutations. In addition, the tumor microenvironment consists of fibroblasts and myeloid cells, which produce multiple inflammatory mediators including DAMPs.[6] However, it remains largely unclear whether inflammatory mediators activate innate immunity by releasing "immunogenic" nucleic acids in the tumor microenvironment. Thus, it is critical to address the molecular mechanisms by which tumor microenvironments can affect the ability of nucleic acids to interact with the pro-inflammatory signal machinery and activate the innate immune system.

TIM-3 is upregulated on Type 1 T helper CD8+ T lymphocytes during the chronic phase of infection as well as during oncogenesis and can trigger their apoptotic demise following the ligation of galectin 9.[7] We identified an unexpected function of TIM-3 in negatively regulating nucleic acid-mediated innate immune responses from DCs found in the tumor microenvironment.[8] TIM-3 is expressed on tumor-infiltrating DCs at much higher levels than on DCs in normal tissues, and preferentially binds with the major DAMP high mobility group box-1 (HMGB1), which has a critical role in stimulating nucleic acid-mediated innate immunity.[9] TIM-3 negatively regulates the HMGB1-mediated recruitment of nucleic acids to the endosomal compartment of DCs, thus shutting down the downstream signaling cascades mediated by TLRs and cytosolic sensors.[8] TIM-3 on DCs thereby enables tumors to evade immunosurveillance by

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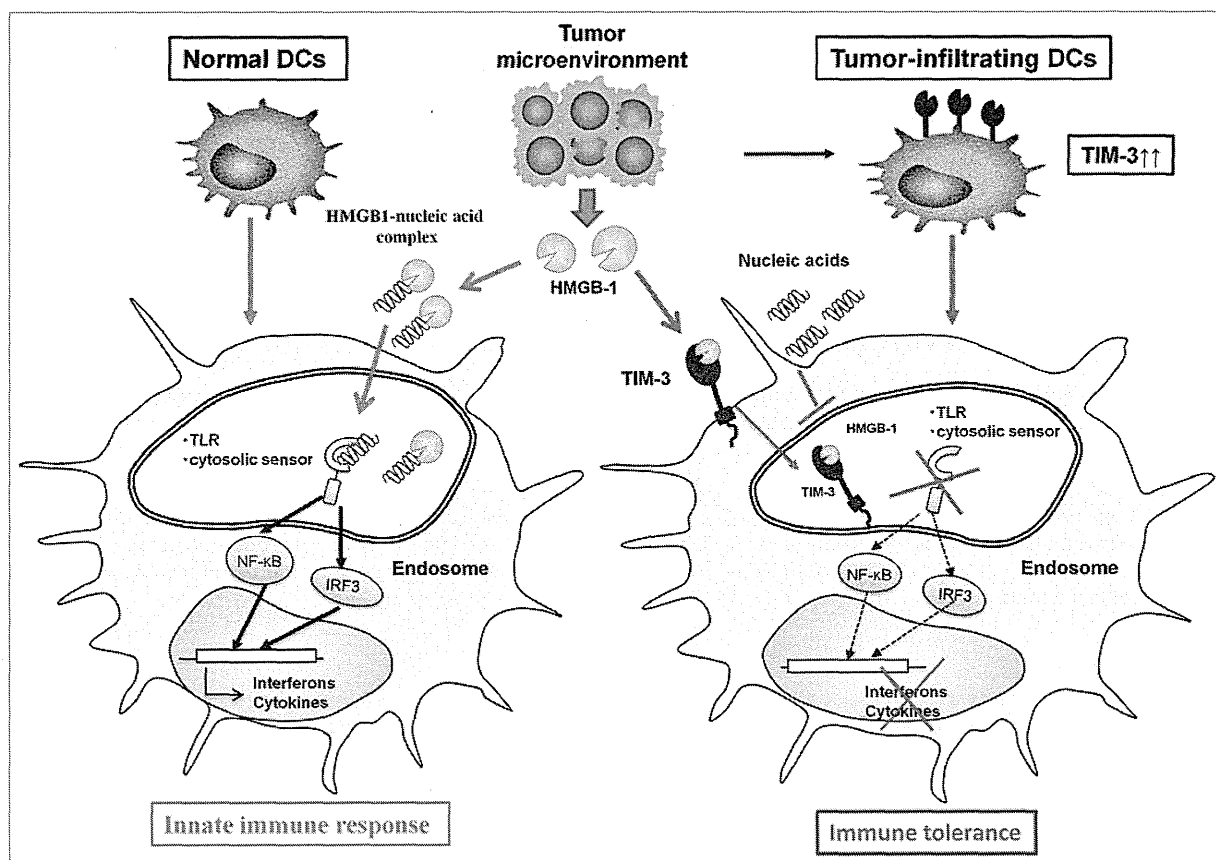

Figure 1. This scheme illustrates the molecular machineries whereby TIM-3 on dendritic cells (DCs) negatively regulates innate immune signals that would be activated by endogenous danger signals under normal conditions. Tumor microenvironments frequently generate endogenous danger signals including HMGB1 due to smoldering inflammation. HMGB1 binds nucleic acids and facilitates the interaction of nucleic acids with pattern recognition receptors including Toll-like receptors (TLRs) and cytosolic sensors, activating innate immune signals. However, tumors counteract innate immunity by upregulating TIM-3 on tumor-infiltrating DCs. The interaction between TIM-3 and HMGB1 inhibits the recruitment of nucleic acids into the endosomal compartment of DCs. Thus, the interaction between TIM-3 and HMGB1 serves as an evasion strategy used by tumors to escape immunosurveillance.

attenuating the sensing of nucleic acids that is potentially triggered by tumor-associated inflammation (**Fig. 1**).

Why TIM-3 on DCs is preferentially recognized by HMGB1 rather than galectin 9 remains largely obscure, but multiple tumor-derived mediators may regulate the repertoires of endogenous danger signals that contribute to the creation of inflammatory tumor microenvironments. Interestingly, a recent report revealed that pattern recognition receptor ligands including DAMPs may serve as driving forces that trigger the release of exogenous HMGB1 from the nucleus of host cells.[10] This suggests that DNA vaccination or endogenous nucleic acids released from dying cells upon cytotoxic chemotherapy may increase the presence of HMGB1 in the tumor microenvironment, thus contributing to the generation of HMGB1-DNA complexes and the activation of innate immunity. With regard to this, tumors appear to utilize TIM-3 on tumor-infiltrating DCs as a means to evade innate immune responses elicited by anticancer therapeutic regimens.

A deeper understanding of the mechanisms linking innate immunity to tumor-associated inflammation and of the impact of negative regulatory pathways established by the tumor microenvironment will provide new strategies to promote endogenous antitumor immune responses and improve the efficacy of anticancer therapy.

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ORIGINAL ARTICLE

Combined blockade of TIM-3 and TIM-4 augments cancer vaccine efficacy against established melanomas

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Abstract Cancer vaccines have been developed to instruct the endogenous immune responses to autologous tumors and to generate durable clinical responses. However, the therapeutic benefits of cancer vaccines remain insufficient due to the multiple immunosuppressive signals delivered by tumors. Thus, to improve the clinical efficacy of cancer immunotherapy, it is important to develop new modalities to overcome immunosuppressive tumor microenvironments and elicit effective antitumor immune responses. In this study, we show that novel monoclonal antibodies (mAbs) specifically targeting either T cell immunoglobulin mucin protein-3 (TIM-3) or T cell immunoglobulin mucin protein-4 (TIM-4) enhance the therapeutic effects of vaccination against established B16 murine melanomas. This is true for vaccination with irradiated B16 melanoma cells engineered to express the flt3 ligand gene (FVAX). More importantly, combining anti-TIM-3 and anti-TIM-4 mAbs markedly increased vaccine-induced antitumor responses against established B16 melanoma. TIM-3 blockade mainly stimulated antitumor effector activities via natural killer cell-dependent mechanisms, while $CD8^+$ T cells served as the main effectors induced by anti-TIM-4 mAb. Our findings reveal that therapeutic manipulation of TIM-3 and TIM-4 may provide a novel strategy for improving the clinical efficacy of cancer immunotherapy.

Keywords TIM-3 · TIM-4 · Cancer vaccines · NK cells · $CD8^+$ T cells · Melanoma

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Introduction

Cancer vaccines have been developed with the aim of efficiently inducing tumor-specific cytotoxic lymphocytes and controlling tumor growth [1, 2]. However, the clinical efficacies of cancer vaccines remain unsatisfactory at present, as hostile tumor microenvironments exploit multiple strategies to counter antitumor immune responses induced by cancer vaccines [3, 4]. Thus, new strategies to overcome immunosuppressive barriers and improve the therapeutic effects of cancer vaccines are urgently needed.

Tumor microenvironments suppress tumor-specific immune responses and impair tumor immunosurveillance by coordinating with negative checkpoint regulators expressed on tumor-infiltrating lymphocytes [5, 6] such as cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), BTLA (B and T lymphocyte attenuator) or T cell immunoglobulin mucin protein-3 (TIM-3) [7–12]. Indeed, the recent clinical success of anti-CTLA-4 and anti-PD-1 monoclonal antibodies (mAbs) in vastly improving the prognosis and survival of patients with advanced cancers has substantiated the major impact of these inhibitory pathways in the negative regulation of antitumor immune responses and clinical prognosis [13–15]. Thus, it is critical to address whether therapeutic manipulation of immune checkpoint regulators other than CTLA-4 or PD-1 can

control tumor growth and enhance antitumor immune responses.

TIM-3 is a key checkpoint receptor responsible for T cell exhaustion, which arises during the chronic phase of infections and cancer, and blockade of TIM-3 restores the antigen-specific effector activities of $CD8^{+}$ T lymphocytes [16, 17]. Pharmacological targeting of TIM-3 increased tumor-specific immune responses and enhanced efficient control of tumor burden, implying that TIM-3 is a potential candidate for reversing immune tolerance and restoring antitumor immune responses within tumor microenvironments [12].

T cell immunoglobulin mucin protein-4 (TIM-4) is a phosphatidylserine receptor that promotes phagocytosis of apoptotic cells [18, 19]. Recent analysis of TIM-4-deficient mice has demonstrated that TIM-4 is critical for the repression of inflammation and maintenance of tolerance [20]. These findings raise the possibility that manipulation of TIM-3 and/or TIM-4 may have an impact on immune responses in tumor microenvironments.

In this study, we demonstrate that the pharmacological blockade of TIM-3 and/or TIM-4 using mAbs stimulates distinct antitumor effector cells within tumor microenvironments. Treatment with anti-TIM-3 mAb increased the numbers and activity of tumor-infiltrating natural killer (NK) cells, whereas anti-TIM-4 mAb recruited mainly $CD8^{+}$ T cells as the source of antitumor activities. Moreover, a combined treatment with anti-TIM-3 and anti-TIM-4 mAbs further increases the efficacy of cancer vaccines as compared to either mAb alone by increasing the numbers and effector functions of both NK cells and $CD8^{+}$ T cells in tumors. These findings imply that pharmacological targeting of TIM-3 and TIM-4 provides a new strategy for improving the antitumor efficacies of cancer vaccines.

Materials and methods

Mice

C57BL/6 mice (6–8-week-old females) were purchased from SCL. OT-I mice were kindly provided by Dr. Shigeo Koyasu (Keio University) and used as described previously [21]. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University.

Cell lines

B16-F10 melanoma cells were obtained from the American Tissue Culture Collection (ATCC). B16-Flt3L cells were kindly provided by Dr. James P. Allison (MSKKC, USA) and used as described previously [22, 23]. B16-OVA cells were kindly provided by Dr. Heiichiro Udono (Okayama University, Japan). The cell lines used in experiments were routinely authenticated by the Central Institute for Experimental Animals (Kawasaki, Japan) for interspecies and mycoplasma contamination by PCR.

Antibodies

Antimouse TIM-3 (RMT3-23) and antimouse TIM-4 (RMT4-53) mAbs were prepared as described previously [23, 24]. The antimouse NK1.1 (PK136), antimouse CD3ε (145-2C11), antimouse CD8α (53-6.7), antimouse CD69 (H1.2F3) and antimouse CD44 (IM7) mAbs were purchased from Biolegend.

In vivo antitumor activities of FVAX vaccines

Mice were injected in the flank subcutaneously at day 0 with 1×10^{5} live B16-F10 melanoma cells and treated on days 3, 5 and 7 with subcutaneously 1×10^{6} irradiated (150 Gy) B16-Flt3L cells in the contralateral flank and intraperitoneal injection of 250 μg of anti-TIM-3 and/or anti-TIM-4 mAbs. Where indicated, depletion of NK cells or $CD8^{+}$ T cells was achieved by two 250-μg injections of anti-NK1.1 mAbs (PK136) or anti-CD8α mAbs (52–6.7) for $CD8^{+}$ T cell depletion on days −2 and −4. Tumor size was measured on the indicated days.

Tumor infiltration/activation marker analysis

Tumor-infiltrating lymphocytes were isolated as described previously [21]. In brief, established tumors were excised from mice vaccinated with FVAX, and single-cell suspensions obtained from the excised tumors were stained with αNK1.1, αCD3ε and αCD69 for NK cells, or with αCD8α, αCD3ε and αCD44 for $CD8^{+}$ T cells to assess the frequencies and activation status. Stained samples were analyzed on a flow cytometer (BD Bioscience).

Cytotoxic assay of intratumor lymphocytes

The established tumors (25 mm^{2}) from B16-OVA cells inoculated subcutaneously into C57BL/6 wild-type mice were treated with FVAX in the presence of control Ig, anti-TIM-3 mAb, anti-TIM-4 mAb or anti-TIM-3 mAb and anti-TIM-4mAb. $CD45^{+}$ lymphocytes were isolated from tumor tissues of the mice 4 days after final treatment. B16 or B16-OVA target cells were incubated with bulk intratumor lymphocytes or those depleted of NK1.1 or TCR-Vβ5 populations by flow cytometry for 6 h at 100:1 of effector to target ratios. Supernatants were collected and subjected to LDH release assay. The maximum or spontaneous release was defined as the counts that emerged